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TECHNICAL MANUSCRIPT 508

# EFFECT OF FORMALDEHYDE ON STAPHYLOCOCCAL ENTEROTOXIN B: IMMUNOGENIC ACTIVITY IN MACACA MULATTA

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MARCH 1969



DEPARTMENT OF THE ARMY
Fort Detrick
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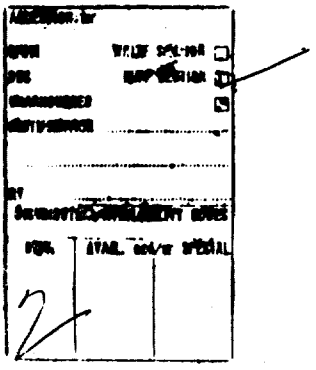

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# EFFECT OF FORMALDEHYDE ON STAPHYLOCOCCAL ENTEROTOXIN B: IMMUNOGENIC ACTIVITY IN *MACACA MULATTA*

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Project 1B562602A059

March 1969

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council.

## ABSTRACT

Monkeys were immunized with enterotoxin or enterotoxoid by intracutaneous injection or by feeding. Identical schedules were used in order to compare the effectiveness of the two antigens and the two routes. Enterotoxin administered intracutaneously was most effective; oral administration of enterotoxoid was least effective. Intracutaneous injection of toxoid and oral feeding of toxin were intermediate and not too dissimilar from each other in effectiveness. Antibody titers and protection persisted for at least 1 year at a relatively high level. Monkeys that had preimmunization hemagglutinins showed an anamnestic response following immunization. The development of protection and the appearance of antibodies subsequent to feeding toxin or toxoid suggest that ingestion of food contaminated by staphylococci or their metabolites may be one cause for the appearance of antitoxin in the serum of supposedly unexposed animals and man.

## I. INTRODUCTION*

We previously reported[1] that 0.3% formaldehyde decreased the immunochemical activity of staphylococcal enterotoxin B and greatly decreased the lethal activity in monkeys within the first 48 hours of exposure without significant effect on the emesis-producing properties. We also observed that (i) both toxoid and toxin were immunogenic in rabbits to the same degree; (ii) both antitoxoid and antitoxin protected monkeys equally well against the effect of toxin; and (iii) antitoxoid and antitoxin neutralized the synergistic effect of enterotoxin for the gram-negative lipopolysaccharide endotoxin in mice.[2] In the present report we compare the immunogenic effect of toxin and toxoid for monkeys when administered by either the intracutaneous or oral route. Bergdoll[3] reviewed earlier studies on immunization with either culture filtrates treated with formalin or untreated filtrates taken by mouth. Evidence of protection was obtained but assay was difficult because both purified enterotoxin and serological assay methods were lacking. In his own work, Bergdoll used partially purified enterotoxin (20% purity), treated with 0.7% formalin and adsorbed to aluminum hydroxide, to immunize monkeys. Effectiveness of the immunization scheme was based on the detection of antibodies by gel diffusion and by challenge with enterotoxin.

## II. MATERIALS AND METHODS

Sixty rhesus monkeys (Macaca mulatta) were conditioned in the laboratory for 2 weeks and closely examined for outward signs of disease prior to the experiment. All animals were bled and tested for antienterotoxin B antibodies before use. Except for a few monkeys, all those that showed the presence of antibodies by hemagglutination (HA) were excluded from the study.

Purified enterotoxin B[4] was diluted in 0.02 M phosphate buffered saline (PBS), pH 7.3, for administration to the monkeys. Enterotoxoid was prepared by dissolving 100 mg of the toxin in 25.0 ml of 0.8% formalin in PBS, pH 8.0, and incubating at 37 C for 18 days. The toxin and toxoid were diluted to the desired concentrations in PBS, pH 7.3, prior to administration.

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Monkeys about 2.5 kg in weight were divided at random into four groups of 15 animals each and immunized over an 8-week period with either enterotoxin B or its toxoid, using identical schedules for both antigens. The toxin was administered either by intracutaneous injection into the posterior of the thigh or by means of a stomach tube. For injection the dose was prepared in 0.25 ml PBS, while 5.0 ml was used for the oral procedure. The initial dose was 2.0 µg per kg body weight. Ten µg/kg were administered at 1 week, 50 µg/kg at 2 weeks, 250 µg/kg at 4 weeks, and 350 µg/kg at each of the 7th and 8th weeks. The total amount of antigen given over the 8-week period was approximately 2.5 mg per animal. Blood samples were obtained from each animal prior to each administration of antigen and prior to challenge. Antitoxin titers were determined by hemagglutination of sheep red blood cells to which enterotoxin was coupled via bisdiazotized benzidine as described by Gordon, Rose, and Sehon.[5]

Three weeks after completion of immunization, the four groups of monkeys were randomly divided into three subgroups of five animals each and challenged with 25, 125, or 625 µg/kg of purified enterotoxin B for each group of five animals. Large numbers of animals had been tested previously with the same enterotoxin preparation and under the same laboratory conditions used in these experiments, and the $LD_{50}$ was determined to be 25 µg/kg.[4] Thus, 1, 5, and 25 $LD_{50}$ doses were chosen for challenge. The challenge dose was given intravenously and the monkeys were observed 5 hours for emesis and 5 days for death. Survivors were randomly assigned to three groups, and at 5, 8, and 11 months after the original challenge one group of survivors was selected for rechallenge with 625 µg/kg of enterotoxin B intravenously. At the time of each challenge the HA antibody titer was established for each animal in order to determine the persistence of the titer over a long period of time.

## III. RESULTS

During the early stages of immunization, injections of enterotoxin caused emesis, and the monkeys showed signs of intoxication. About 35% of the animals vomited after the first two injections. No responses occurred subsequently. About 20% of the animals that received toxin orally showed a similar response following the first two doses. None of the animals that received toxoid showed any signs of intoxication during immunization.

Figure 1 illustrates the development of the HA titers in the monkeys during the immunization period prior to challenge. The figures given for each point are mean values for the 15 animals in individual groups; those animals with some demonstrable titer at the beginning of the experiment were considered separately. HA titers developed most quickly and to the highest

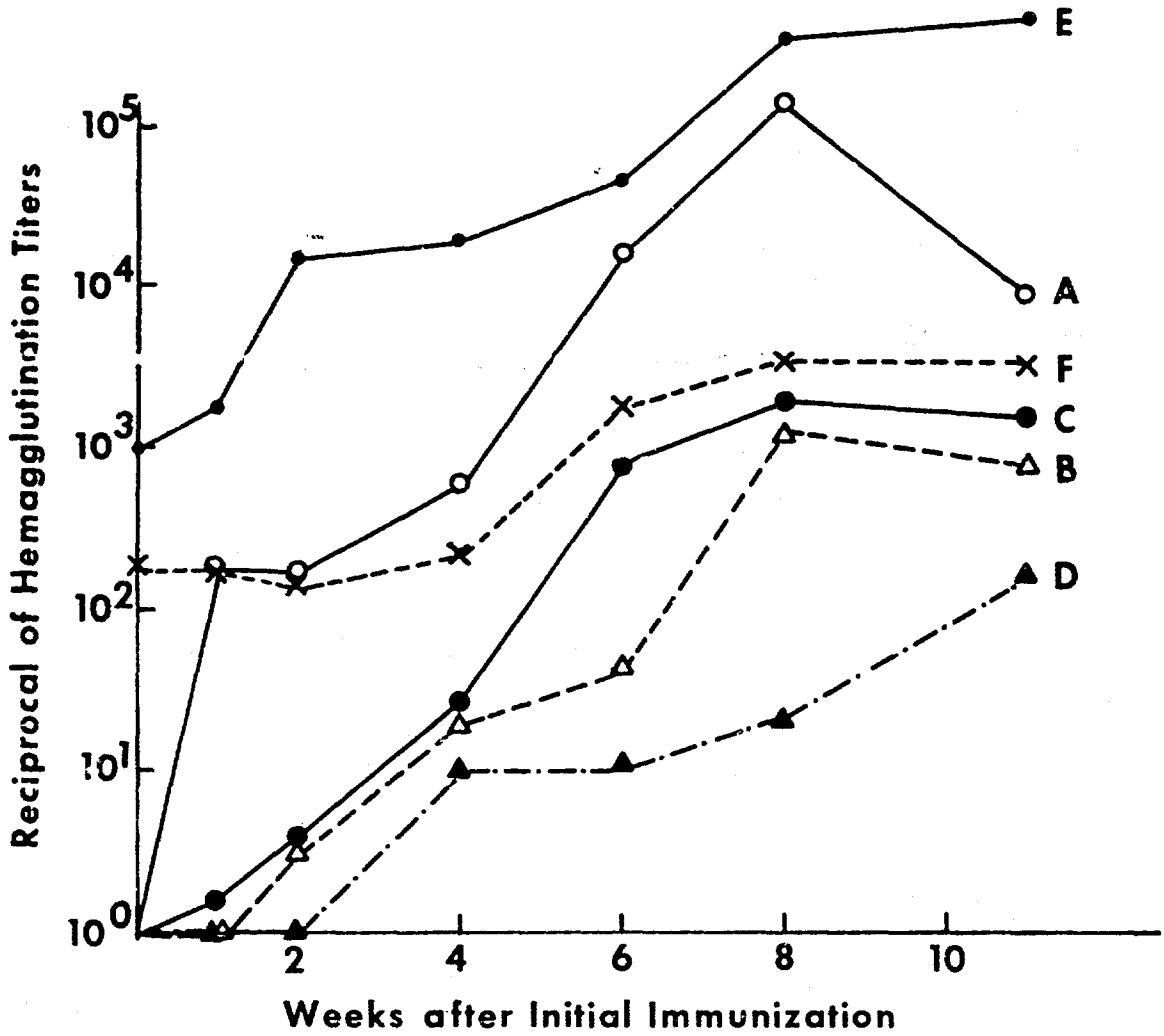


FIGURE 1. Mean HA Titers in Immunized Monkeys. A, with enterotoxin via intracutaneous injection; B, with enterotoxin by feeding; C, with enterotoxoid by intracutaneous injection; D, with enterotoxoid by feeding; E, mean titers of monkeys that showed some serum antibodies prior to intracutaneous injection of enterotoxin; F, mean titers of monkeys that showed preimmunization hemagglutinins and that received toxoid orally. Values are reciprocals of end point titers; all data are means for 15 animals.

level in monkeys injected intracutaneously with unaltered toxin. Maximum titers in these animals were observed on the 56th day and some decrease was noted by the 77th day. Animals injected with toxoid responded more slowly and with lower titers. The hemagglutinin response to oral immunization was relatively poor, especially among the monkeys given toxoid via stomach tube. The immune response was delayed after feeding either toxin or toxoid, titers were lower and, in fact, attained only a mean value of about 1:120 (range 1:40 to 1:640). The anamnestic response of those monkeys whose sera reacted prior to immunization and who received toxin by injection was unrelated to the preimmunization titer. The latter ranged from 1:10 to 1:2,560, but 1 week after the initial injection all four monkeys showed titers of 1:2,560 (Table 1, Fig. 1). On the other hand, the initial anamnestic response of those monkeys fed toxoid appeared to be related to the preimmunization HA titer.

Table 2 shows results of the challenge of the four immunized groups with 1-, 5-, and 25-$LD_{50}$ amounts of enterotoxin B. It also shows the mean HA titer of each group prior to challenge. The monkeys that were immunized with enterotoxin by intracutaneous injection demonstrated the most protection against challenge at all dose levels. Neither emesis nor death was observed among these animals, even at the highest challenge dose, 625 µg (25 $LD_{50}$) per kg body weight. No deaths occurred in the group that was injected with toxoid, but two of five monkeys vomited following challenge with 125 µg per kg and five of five animals vomited after receiving 625 µg per kg. Those animals immunized by feeding responded most poorly. Among those that received toxin by this route, the ematic dose ($ED_{50}$) was about 25 µg, and the $LD_{50}$ approximated 625 µg. Following toxoid immunization by the oral route, three monkeys succumbed to challenge with 125 µg per kg, and two of five died following challenge with 625 µg. For monkeys not previously exposed to enterotoxin, the $ED_{50}$ is about 0.3 µg per kg body weight and the $LD_{50}$ is 24.0 µg per kg body weight.

The relationship between protection and HA titer can be expressed in only a general way. In the group that did not respond to challenge, those animals immunized with enterotoxin by the intracutaneous route, the titers were relatively high at the time of challenge. They ranged from 1:20,480 through 1:1,310,720. Those monkeys that were injected with enterotoxoid developed titers ranging from 1:640 to 1:5,120, which were not sufficient to prevent an emetic response to the higher challenge doses but were sufficient to prevent death. Among the animals immunized by the oral route, the titers varied from <1:10 through 1:10,240 and the relationship between the HA titers and protection is less clear. No deaths occurred among the monkeys challenged with 1 $LD_{50}$, even though several of the animals failed to show antibodies at a 1:10 dilution. Following challenge with 5 or 25 $LD_{50}$, deaths occurred among the animals with serum titers of 1:1,280 or less; however, emesis occurred among monkeys showing a wide range of serological responses.

TABLE 1. ANAMNESTIC RESPONSE OF MONKEYS WITH PREIMMUNIZATION ANTIENTEROTOXIN B HEMAGGLUTININS

| Monkey No. | Immunization | HA Titer (Reciprocal of Dilution) on Indicated Day | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 0 | 7 | 14 | 28 | 42 | 56 | 77 |
| E449 | Enterotoxin intracutaneous | 320 | 2,560 | 5,120 | 20,480 | 81,920 | 655,360 | 327,680 |
| E456 | Enterotoxin intracutaneous | 10 | 2,560 | 20,480 | 40,960 | 81,920 | 163,840 | 163,840 |
| E476 | Enterotoxin intracutaneous | 2,560 | 2,560 | 40,960 | 40,960 | 81,920 | 655,360 | 655,360 |
| E4123 | Enterotoxin intracutaneous | 1,280 | 2,560 | 2,560 | 10,240 | 10,240 | 655,360 | 1,310,720 |
| E164 | Enterotoxoid, oral | 160 | 160 | 80 | 160 | 640 | 1,280 | 2,560 |
| E22 | Enterotoxoid, oral | 40 | 20 | 40 | 320 | 2,560 | 5,120 | 2,560 |
| E213 | Enterotoxoid, oral | 640 | 640 | 320 | 640 | 5,120 | 10,240 | 10,240 |

TABLE 2. RESPONSE OF IMMUNIZED MONKEYS[a] TO INTRAVENOUS CHALLENGE WITH ENTEROTOXIN B

| | Challenge Dose | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 25 μg/kg | | | 125 μg/kg | | | 625 μg/kg | | |
| | Mean HA Titer[b] | Response[c] Emesis | Death | Mean HA Titer | Response Emesis | Death | Mean HA Titer | Response Emesis | Death |
| Toxin injected | 45,000 | 0 | 0 | 557,000 | 0 | 0 | 98,000 | 0 | 0 |
| Toxoid injected | 2,300 | 0 | 0 | 1,500 | 2 | 0 | 1,300 | 5 | 0 |
| Toxin oral | 900 | 2 | 0 | 1,100 | 5 | 0 | 700 | 4 | 3 |
| Toxoid oral | 500 | 4 | 0 | 150 | 5 | 3 | 2,700 | 3 | 2 |

a. Five animals per group.
b. HA - reciprocal of hemagglutinating titer.
c. Emesis within 5 hours; death within 5 days.

Protection persisted among these monkeys for at least 1 year. Groups of animals that survived the initial challenge were rechallenged 5, 8, and 11 months later with 25 $LD_{50}$ (625 μg per kg body weight). The results are tabulated in Table 3. At 5 months, 33% of all animals rechallenged vomited and 11% succumbed to enterotoxin; after 8 months, 70% showed emesis and 23% died; and at 11 months 57% vomited and 7% died. At the time of the last exposure, the sera of five of the 14 monkeys still agglutinated enterotoxin-sensitized red blood cells at titers ranging from 1:640 through 1:328,000. The mean value of antibody titers for the animals tested at this period is shown in Table 4.

TABLE 3. MONKEY RESPONSE TO RECHALLENGE WITH 25 $LD_{50}$ ENTEROTOXIN B 5, 8, AND 11 MONTHS AFTER INITIAL CHALLENGE

| | Time of Challenge[a] | | | | | |
|---|---|---|---|---|---|---|
| | 5 Months | | 8 Months | | 11 Months | |
| | Emesis | Death | Emesis | Death | Emesis | Death |
| Toxin intracutaneous | 0/3 | 0/3 | 2/5 | 0/5 | 0/3 | 0/3 |
| Toxoid intracutaneous | 2/3 | 1/3 | 2/4 | 2/4 | 3/4 | 1/4 |
| Toxin oral | 0/2 | 0/2 | 4/4 | 1/4 | 3/4 | 0/4 |
| Toxoid oral | 1/1 | 0/1 | 4/4 | 1/4 | 2/3 | 0/3 |
| Controls | 2/2 | 2/2 | 3/4 | 1/4 | ND[b] | ND |

a. Months after initial challenge.
b. ND = no data.

TABLE 4. PERSISTENCE OF HEMAGGLUTININ TITERS IN MONKEYS FOLLOWING IMMUNIZATION AND CHALLENGE WITH ENTEROTOXIN B

| | Mean Titer[a] | | |
|---|---|---|---|
| | 5 Months[b] | 8 Months | 11 Months |
| Toxin intracutaneous | 23,000 | 127,000 | 123,000 |
| Toxoid intracutaneous | 7,700 | 7,800 | Negative |
| Toxin oral | 2,600 | 66,000 | 640 |
| Toxoid oral | 300 | 5,300 | 1,900 |
| Range | 160 to 41,000 | ND[c] | <10 to 328,000 |

a. Reciprocal of end point dilution.
b. Months after initial challenge.
c. ND = no data.

## IV. DISCUSSION

These experiments show that for the monkey, enterotoxin B was a more effective protective antigen than was formaldehyde-treated toxoid. This was so regardless of the route of immunization used. When injected with either toxin or toxoid by the intracutaneous route, monkeys were protected against death even when challenged with 25 $LD_{50}$. Complete protection against both emesis and death was obtained only when enterotoxin was injected. Regardless of the antigen or the route, protection against death following this challenge dose persisted at a relatively high level for at least 1 year; protection against emesis tapered off gradually but was still at a significant level when the animals were exposed to 625 µg of enterotoxin per kg body weight, approximately 2,000 times the emetic dose.

The protection resulting from the various immunization procedures was paralleled by the appearance of hemagglutinins; the optimal procedure for protection was also optimal for antibody production. It appears that parenteral injection of enterotoxin results in better protection than does injection of toxoid. This is unlike the results we reported previously, which showed no significant difference between toxin and toxoid when injected into rabbits.[1] Both types of rabbit antisera (antitoxin and antitoxoid) were equally protective.

The immunization procedure used here was not optimal. Dosages were small and administered over a relatively long time. This was necessary because of the response of monkeys to the toxin. Enterotoxoid can be administered in much greater doses and, with the use of adjuvants such as aluminum hydroxide, would very likely result in more efficient immunization.

The anamnestic response observed with monkeys that had some antitoxin antibodies in their serum prior to immunization emphasizes the necessity of testing animals prior to use. We have observed that from 4.0 to about 50.0% of the monkeys tested prior to exposure show the presence of antibodies. Although titers are generally below 1:80, they may be as high as 1:2,500 in some cases. These experiments in which antibodies were induced by oral administration suggest one way by which animals may become immunized in nature. Minor staphylococcal infections, or even staphylococci found as normal inhabitants of mucous surfaces, may contribute.

The persistence of titers in all groups for at least 1 year after challenge was undoubtedly influenced by the intravenous injection administered for the initial challenge. Whether this explains the similarity in response among the animals in all groups after 11 months can only be conjectured. We found a tenfold decrease in immunity after 1 year (Table 3) that, however, was not as marked as Bergdoll observed.

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Unclassified
Security Classification

**DOCUMENT CONTROL DATA - R & D**
*(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)*

| 1. ORIGINATING ACTIVITY (Corporate author) | 2a. REPORT SECURITY CLASSIFICATION |
|---|---|
| Department of the Army<br>Fort Detrick, Frederick, Maryland, 21701 | Unclassified |
| | 2b. GROUP |

3. REPORT TITLE
EFFECT OF FORMALDEHYDE ON STAPHYLOCOCCAL ENTEROTOXIN B: IMMUNOGENIC ACTIVITY IN MACACA MULATTA

4. DESCRIPTIVE NOTES *(Type of report and inclusive dates)*

5. AUTHOR(S) *(First name, middle initial, last name)*
Sidney J. Silverman
Gary T. Moore
William G. Roessler

| 6. REPORT DATE | 7a. TOTAL NO. OF PAGES | 7b. NO. OF REFS |
|---|---|---|
| March 1969 | 17 | 5 |

| 8a. CONTRACT OR GRANT NO. | 9a. ORIGINATOR'S REPORT NUMBER(S) |
|---|---|
| b. PROJECT NO. 1B562602A059 | Technical Manuscript 508 |
| c. | 9b. OTHER REPORT NO(S) *(Any other numbers that may be assigned this report)* |
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13. ABSTRACT

Monkeys were immunized with enterotoxin or enterotoxoid by intracutaneous injection or by feeding. Identical schedules were used in order to compare the effectiveness of the two antigens and the two routes. Enterotoxin administered intracutaneously was most effective; oral administration of enterotoxoid was least effective. Intracutaneous injection of toxoid and oral feeding of toxin were intermediate and not too dissimilar from each other in effectiveness. Antibody titers and protection persisted for at least 1 year at a relatively high level. Monkeys that had preimmunization hemagglutinins showed an anamnestic response following immunization. The development of protection and the appearance of antibodies subsequent to feeding toxin or toxoid suggest that ingestion of food contaminated by staphylococci or their metabolites may be one cause for the appearance of antitoxin in the serum of supposedly unexposed animals and man.

14. Key Words

*Formaldehyde
*Staphylococcal enterotoxin B
*Monkeys
Immunology
Enterotoxin
Intradermal injections
Antibodies
Hemagglutination
Staphylococcal infections

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